Angiogenic capacity of M1- and M2-polarized macrophages is determined by the levels of TIMP-1 complexed with their secreted proMMP-9

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Supplemental Data

Neutrophil and monocyte isolation

To isolate neutrophils, EDTA-containing blood was diluted 1:6 with 6% solution of a 500-kDa Dextran (Spectrum Chemical, Gardena, CA). The white blood cell fraction was harvested after 20-min sedimentation of erythrocytes at ambient temperature. The leukocyte fraction was diluted 1:1 with HBSS and overlaid onto 1.077 Ficoll-Histopaque (Sigma). Granulocytes were separated by centrifugation of the gradient at 400xg for 40 min at ambient temperature. The cell pellet was exposed to hypotonic shock to remove the erythrocytes. The remaining granulocytes were washed twice with HBSS, counted, and resuspended at the concentrations indicated in the text.

To isolate monocytes, the peripheral blood was diluted 1:2 with 0.9% NaCl and centrifuge at 200xg for 15 min to remove platelets. After removal of the platelet-containing supernatant, the rest of blood was diluted 1:2 with 0.9% NaCl and overlaid on 1.077 Ficoll-Histopaque at 4:1 v:v ratio. Following centrifugation at 400xg for 40 min, mononuclear fraction was collected form the top of a Ficoll-Histopaque layer, and incubated in RPMI 1640 supplemented with 13 mM of sodium citrate in PBS. After incubation for 30 min at 37^0C, the mononuclear fraction was overlaid on Percoll gradient. The gradient was prepared by mixing of 9 volumes of Percoll (Sigma) with 1 volume NaCl 1.5M. This isosmotic Percoll solution was mixed at 1:1 v:v ratio with 13 mM sodium citrate buffer in PBS. The cell-overlaid gradient was centrifuged at 400xg for 30 min at ambient temperature to separate monocytes and lymphocyte populations. The lymphocytes and monocytes were collected respectively from the bottom of the tube and interphase, washed twice with the 13 mM sodium citrate buffer, counted and resuspended in DMEM.

The purity of isolated cell populations was assessed by staining with the Protocol Hema-3 kit (Fisher Diagnostics, Middletown, VA).

Purification of human proMMP-9 and its complexing with TIMP-1

ProMMP-9 was purified from human neutrophil releasates and serum-free (SF) medium conditioned by human monocytic cells (monocytes and mature and polarized macrophages) by gelatin-Sepharose affinity chromatography and analyzed for purity and protein concentrations as described.[1,2] Briefly, Gelatin Sepharose 4B beads (GE Healthcare, Uppsala, Sweden) were washed with 50 mM TBS, pH 7.4, mixed 1:10 (v:v) with neutrophil releasates or SF-CM, and incubated overnight at 4^0C with rotation. Non-bound fraction was collected after centrifugation at 13,000xg for 5 min. The beads were washed twice with excess TBS buffer and bound proteins were eluted with 10% DMSO in TBS. The non-bound and eluted proteins were analyzed by gelatin zymography and silver staining. For complexing with TIMP-1, 100 ng of proMMP-9 was incubated for 2 hr at ambient temperature with 5-fold excess of recombinant TIMP-1. Formed proMMP-9:TIMP-1 complexes were re-purified by gelatin-Sepharose chromatography.

Western blot analysis for MMP-9 and TIMP-1

Following SDS-PAGE of cell CM or purified preparations under reducing or non-reducing conditions, separated proteins were transferred onto a PVDF membrane. Western blot analysis for MMP-9 and TIMP-1 was performed on the membranes cut horizontally at the level of a 50-kDa mol. wt. marker. The upper portions were probed with anti-human MMP-9 mAbs (a mixture of highly specific murine mAbs 8-3H, 7-11C and 6-6B, all generated in our laboratory) or anti-mouse MMP-9 rabbit antibody (ab38898; Abcam). The lower portions of the membranes were probed correspondingly with anti-human TIMP-1 mAb (MAB3300; Millipore) or anti-murine TIMP-1 goat antibody (AF980; R&D Systems).

Gelatin zymography

Samples of CM were collected 48 hr after replacing serum-containing culture medium with SF DMEM. To prepare neutrophil releasate, freshly isolated neutrophils were resuspended in PBS at $1x10^7$ cells per mL and incubated for 2 hr at 37^0C with occasional agitation. The cell ghosts were spun down at 3,200xg at 4^0C for 30 min, and the supernatant (releasate) was collected to provide the source of neutrophil proMMP-9. From 5 to 10 µL of neutrophil releasate and from 10 to 30 µL of CM were loaded per lane of 10% pre-cast gelatin gel (Life Technologies, Carlsbad, CA) in SDS sample buffer containing no DTT. After

electrophoresis, the gels were washed twice in 2.5% Triton X-100 and then incubated at 37^0C in 50 mM Tris buffer, pH 7.5, supplemented with 100 mM NaCl, 5 mM $CaCl_2$, and 0.2% Brij 35. After overnight incubation the gels were stained with 0.25% Coomassie Blue G-250 in 10% acetic acid and destained in distilled water to visualize bands of gelatinolytic activity.

Silver staining of SDS-PAGE gels

A modification of Vorum silver staining protocol was used for silver staining of SDS-PAGE gels.[3] Briefly, the proteins were separated on Novex 4-20% Tris-Glycine gels under non-reduced conditions. After electrophoresis, the gels were fixed overnight in a solution of 50% methanol, 12% acetic acid, and 0.05% formaldehyde. The gels were washed in 35% ethanol for 20 min and then sensitized in 0.02% sodium thiosulfate for 2 min. After 3 washes in water, the gels were stained with 0.2% $AgNO_3$ in 0.076% formaldehyde for 20 min, washed in water, and developed in a mixture of 6% sodium carbonate, 0.05% formaldehyde, and 0.0004% sodium thiosulfate. The developing reaction was stopped with a solution of 50% methanol.

Transfection of human macrophages with siRNA constructs

Human macrophages were transfected with siRNA against *TIMP1* or control siRNA using GeneMute kit from SignaGen Laboratories. The pool of 3 different siRNA constructs against *TIMP1* (sc-29505; Santa Cruz Biotechnology) were used to guarantee the specificity and effectiveness of TIMP-1 downregulation. Importantly, siRNA mixture against *TIMP1* did not cause any significant changes in the levels of proMMP-9 production, indicating the specificity of *TIMP1* downregulation. Control siRNA (sc-37007; Santa Cruz Biotechnology) consists of a scrambled sequence that does not lead to the specific degradation of any cellular message.

Immunofluorescent staining

Human neutrophils, monocytes, and mature and polarized macrophages were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 3 min, washed and introduced to primary antibodies against human MMR (CD206) (#321101; Biolegend) or MMP-9 (mAb 8-3H; generated in our laboratory). Following overnight incubation, the cells were washed and incubated with AlexaFluor488-conjugated secondary antibodies. Immunofluorescent

images for neutrophils, monocytes, and M0- and M1-macrophages were taken at exposure time found optimal for maximal intensity observed in M2-macrophages.

Flow cytometry analysis for cell surface markers

Freshly isolated human neutrophils and monocytes, and mature and polarized macrophages were incubated for 45-60 min on ice in FACScan buffer (PBS/0.5% BSA/0.02% sodium azide) supplemented with 10-20 μg/mL of primary murine mAbs against indicated antigens. After washing, the cells were incubated with the secondary FITC-conjugated goat anti-mouse antibody. BM-derived murine macrophages were pretreated with 10 μg/mL of rat mAb 2.4G2 against CD16/CD32 (Fc Block; Becton Dickinson), to prevent binding of primary antibodies to Fc receptor expressed on monocytic cells. After 10-min incubation on ice, AlexaFluor488-conjugated rat mAbs, BM8 against mouse F4/80 or C068C2 against mouse MMR (CD206) (both from Biolegend), were added to the cells at 10 μg/mL without removal of Fc Block. After washing, the cells were resuspended in FACScan buffer supplemented with 0.3 μg/mL propidium iodide to exclude dead cells from analysis. The levels of MFI were determined in a FACScan flow cytometer by using CellQuest software (Becton Dickinson.)

Quantitative Real Time RT-PCR (qRT-PCR) analysis for gene expression

Total RNA was extracted from the cells with TRIzol (Invitrogen) and 2 μg of isolated RNA was reverse-transcribed using the RNA to cDNA EcoDry Premix (#639549; Clontech). The resulting cDNA was analyzed by qRT-PCR in an I-cycler (Bio-Rad). Each reaction contained 60 ng of cDNA as template, LightCycler 480 SYBR Green Master Mix (#04707516001; Roche), and each of forward and reverse primers (indicated below) used at 0.4 μM. PCR conditions included heating for 5 min at 95^0C, followed by 40 cycles of 30 s at 95^0C, 30 s at 60^0C, and 60 s at 30 s at 72^0C. A melt curve analysis was performed to ensure specific amplification. For each target gene, relative levels of expression were normalized against housekeeping gene signal, generating ΔCt value (ΔCt=Ct target gene – Ct reference gene). Differences in gene expression levels between cell types or culture conditions were calculated according formula $2^{-\Delta Ct}$ as described.[4] Where indicated, the relative gene expression was analyzed in comparison to control condition/cell type according to the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt=ΔCt experimental setting - ΔCt control setting.[5]

Primer Sets for Human Genes

Gene	Encoded Protein (Abbreviation)	Forward Primer	Reverse Primer
ACTB	Beta-actin (β-actin)	CAT CAC CAT TGG CAA TGA GC	CGA TCC ACA CGG AGT ACT TG
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	ACT GCT AGC CGC TTC TTC TT	GAC AAG CTT CCC GTT CTC AG
GUSB	Beta-glucuronidase	CTC ATT TGG AAT TTT GCC GAT T	CCG AGT GAA GAT CCC CTT TTT A
ARG1	Arginase, type I; α-Arginase-1, ARG1	TTC TCA AAG GGA CAG CCA CG	TCA AGC AGA CCA GCC TTT CT
NOS2	Nitric oxide synthase, inducible (iNOS)	GTT CTC AAG GCA CAG GTC TC	GCA GGT CAC TTA TGT CAC TTA TC
MMP9	Matrix metalloproteinase-9 (MMP-9)	TTG ACA GCG ACA AGA AGT GG	GCC ATT CAC GTC CTT AT
MRC1	Mannose receptor, C type 1 (MRC1), Macrophage mannose receptor 1 (MMR), CD206	GCC AAA TGA CGA ATT GTG GA	CAC GAA GCC ATT TGG TAA ACG
TIMP1	Tissue inhibitor of metalloproteinases 1 (TIMP-1)	ACC ACC TTA TAC CAG CGT TAT	GGT GTA GAC GAA CCG GAT GTC

Primer Sets for Murine Genes

Gene	Encoded Protein	Forward Primer	Reverse Primer
ActB	Beta-actin (β-actin)	CTC TGG CTC CTA GCA CCA TGA AGA	GTA AAA CGC AGC TCA GTA ACA GTC CG
Arg1	α-Arginase-1	CAG AAG AAT GGA AGA GTC AG	CAG ATA TGC AGG GAG TCA CC
Nos2	Nitric oxide synthase, inducible (iNOS)	CCC TTC AAT GGT TGG TAC ATG G	ACA TTG ATC TCC GTG ACA GCC
Mmp9	Matrix metalloproteinase 9 (MMP-9)	GCG TGT CTG GAG ATT CGA CTT	GGT CCA CCT TGT TCA CCTC
Mrc1	Mannose receptor, C type 1 (MRC1), Macrophage mannose receptor 1 (MMR), CD206	TCT TTG CCT TTC CCA GTC TCC	TGA CAC CCA GCG GAA TTT C
Tnf	Tumor necrosis factor α (TNFα)	GAC GTG GAA GTG GCA GAA GAG	TGC CAC AAG CAG GAA TGA GA
Ym1	T-lymphocyte-derived eosinophil chemotactic factor (ECF-L)	GGG CAT ACC TTT ATC CTG AG	CCA CTG AAG TCA TCC ATG TC

Supplemental Tables

Supplemental Table S1. Expression of cell surface markers in human monocytes and macrophages

Cell Marker (CD)	Monocytes	M0-macrophages	M1-macrophages	M2-macrophages
Non-specific (Mo IgG)	4.8 (99.8%)	4.7 (99.1%)	5.3 (98.3%)	4.2 (99.2%)
CD80	6.7 (100%)	38.1 (63.1 %)	**67.7 (98.1%)**	24.5 (42.3%)
CD86	6.2 (100%)	**69.1 (94.1%)**	**188.8 (98.3%)**	**145.3 (99.0%)**
CD181 (CXCR1)	8.1 (99.9%)	60.2 (88.7%)	**101.3 (97.7%)**	30.7 (75.1%)
CD206 (MMR)	5.7 (99.96%)	7.6 (99.80%)	7.8 (99.9)%	**87.9 (99.9%)**

Human monocyte-derived macrophages were detached with the enzyme-free buffer and stained with 10 µg/mL of mouse mAbs against human CD80 (clone 2D10), CD86 (clone IT2.2), CD181 (clone 8F1/CXCR1) and CD206 (MMR) (clone 15-2) (all from Biolegend). Mouse IgG (Mo IgG, Sigma) was used as negative control for non-specific binding. The mean immunofluorescence intensity (MFI) for each bound antibody was determined following incubation with FITC-conjugated goat-anti mouse antibody (Sigma). The presented data are MFI and in parentheses, the percentage of cells expressing the indicated MFI.

Supplemental Table S2. Maturation of BM-derived macrophages

Days in culture	Rat IgG	F4/80
1	5 (98.7%)	**77 (88.1%)**
4	5 (99.7)	**133 (55.3)**
6	7 (99.7%)	**590 (99.5%)**
9	11 (99.6%)	**936 (99.6%)**

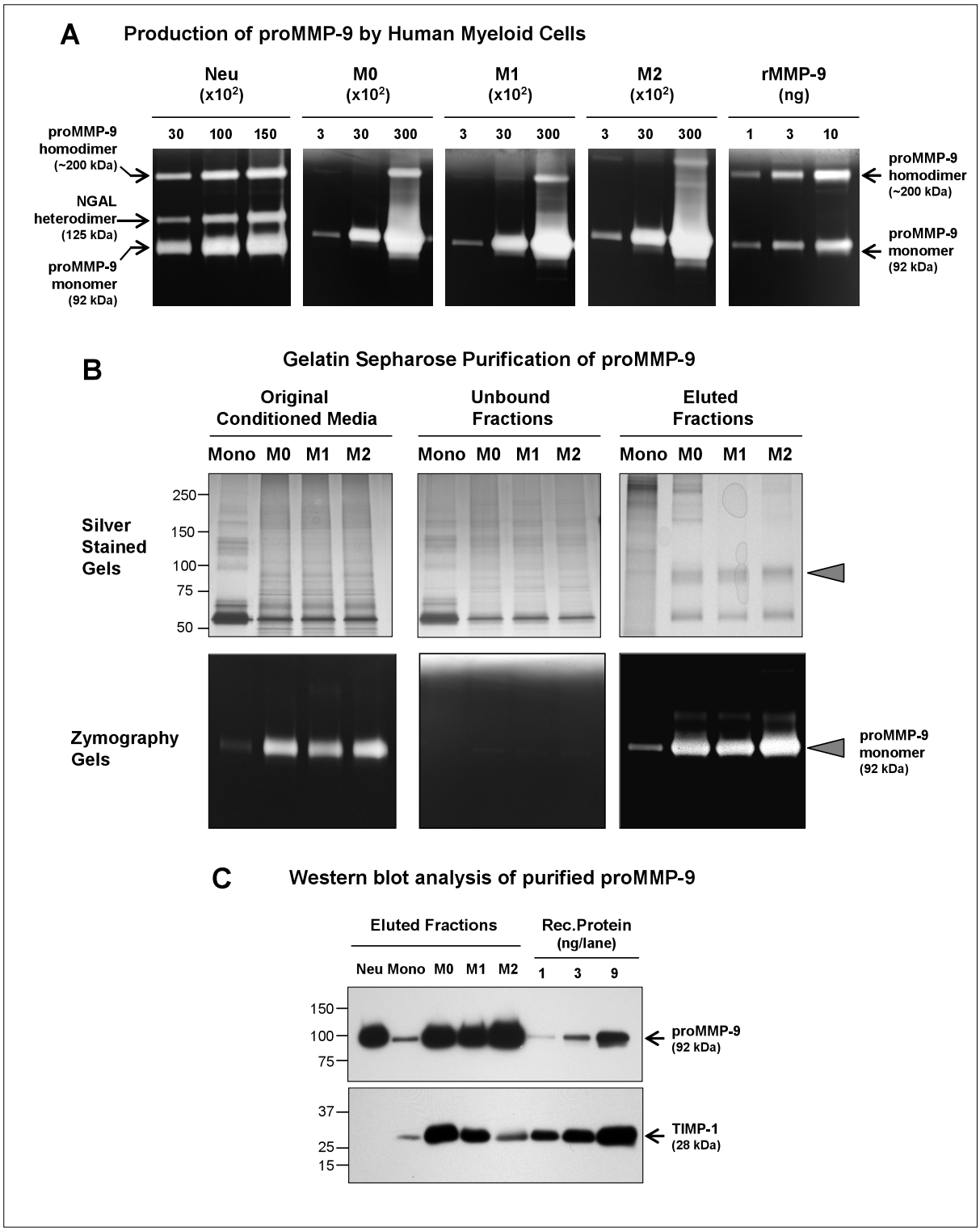
Bone marrow cells from WT C57BL/6 mice were incubated as adherent monolayers in the presence of 10% of L929 CM that provided the source of murine M-CSF. At the indicated time points, the cells were detached with enzyme-free buffer and stained with AlexaFluor488-conjugated rat mAb F4/80. AlexaFluor488-conjugated rat IgG2a served as a negative control. For each time point, the mean fluorescence intensity is presented in bold and the percentage of cell population shift relative to rat IgG control is presented in parentheses.

<u>Supplemental Table S3</u>. Expression of macrophage mannose receptor (MMR, CD206) in murine bone marrow-derived macrophages

Cell Phenotype	Rat IgG	MMR (CD206)
M0	6 (99.7%)	**52 (73.1%)**
M1	6 (98.1)	**41 (35.1)**
M2	6 (97.7%)	**153 (93.2%)**

Bone marrow cells from WT C57BL/6 mice were incubated as adherent monolayers in the presence of murine M-CSF (10% of L929 CM) for 7 days to generate mature M0 macrophages. M0 macrophages were polarized into M1 or M2 phenotypes by exposing the cells to LPS/IFNγ or IL-4, respectively. Following 2-day incubation, the cells were detached with enzyme-free buffer and stained with AlexaFluor488-conjugated rat mAb against murine MMR (CD206). AlexaFluor488-conjugated rat IgG served as a negative control. The mean fluorescence intensity is presented in bold and the percentage of cell population shift relative to rat IgG control is presented in parentheses.

Supplemental Figures and Legends

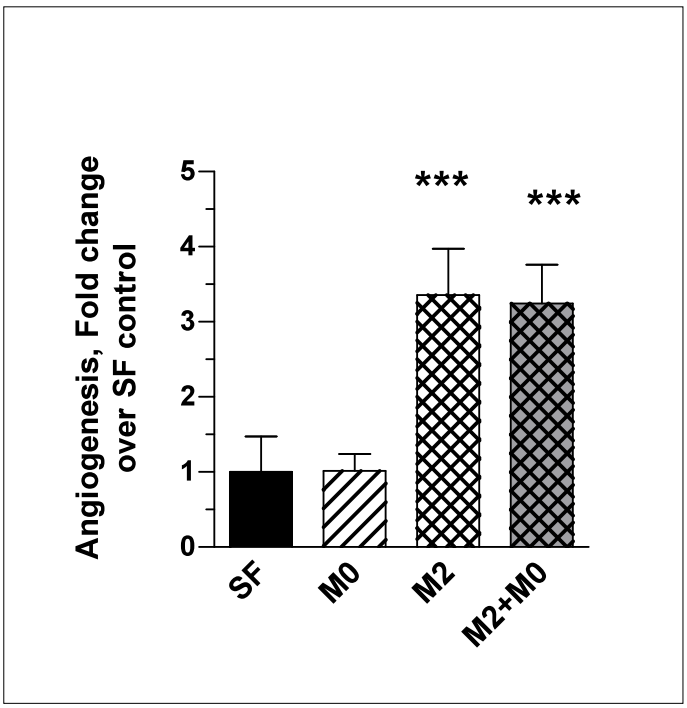


Supplemental Figure S1. Production of proMMP-9 by human myeloid cells, proMMP-9 purification and analysis

(A) Comparative analysis of proMMP-9 production. Human neutrophils (Neu) were induced with PMA to release their secretory granules, and the releasate was collected 30 min later. Monocyte-derived macrophages of M0-, M1-, and M2-phenotypes were incubated in SF medium, and CM collected 48 hrs later. Neutrophil releasate and macrophage CM, generated by the cells at increasing concentration were analyzed by gelatin zymography (the number of cells that generated releasate or CM loaded per gel lane is indicated above corresponding panels), in comparison with the indicated amounts of recombinant human proMMP-9. Analysis of these particular zymograms indicate that within 30 min $1x10^6$ neutrophil release 1.5-2 μg proMMP-9, while macrophages require 48 hr to synthesize and secrete 1.5-2.5 μg proMMP-9.

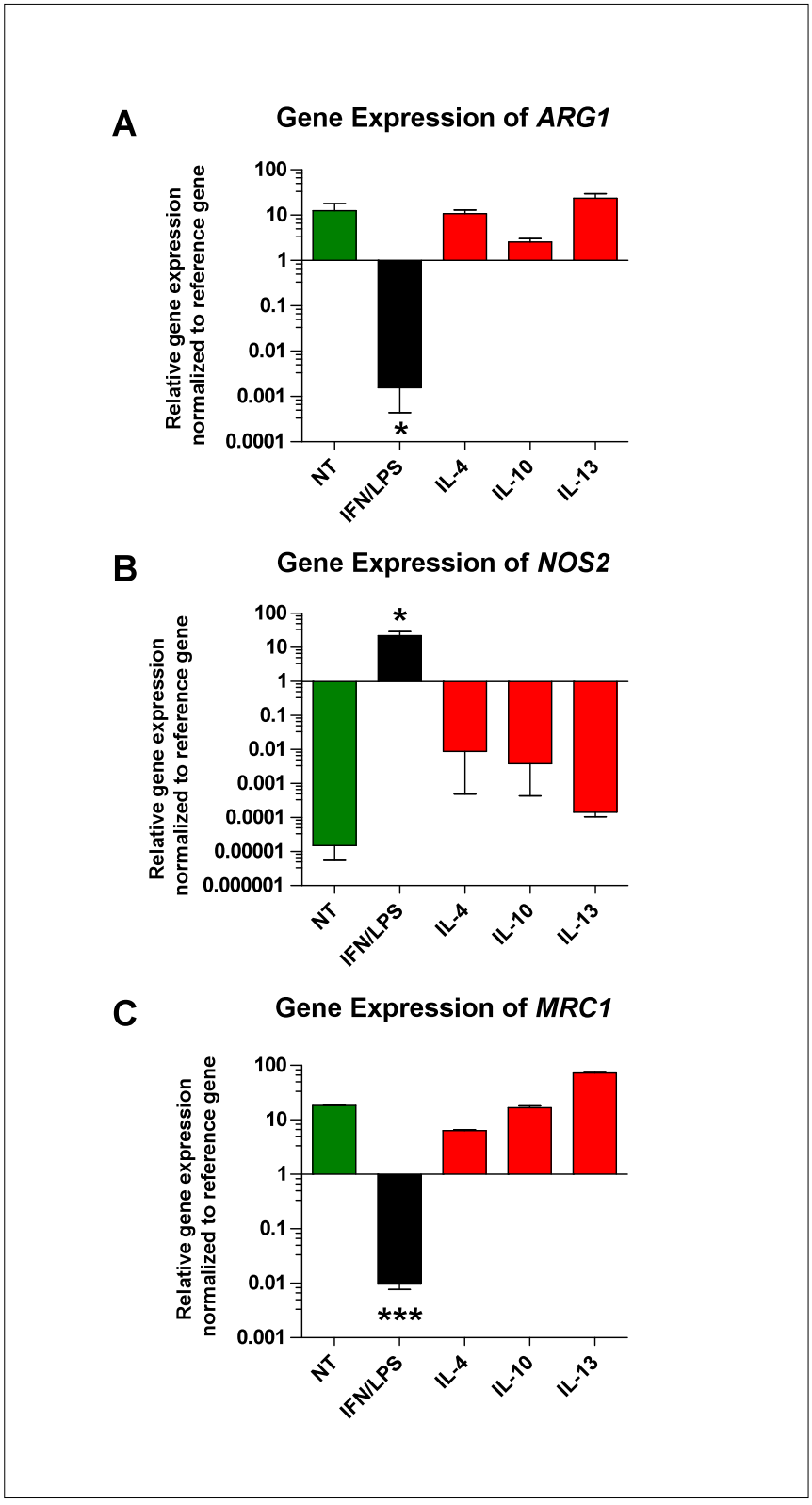
(B) Gelatin-Sepharose purification of proMMP-9 secreted by monocytes and macrophages. SF medium conditioned for 48 hr by freshly isolated monocytes ($1x10^7$ cells/mL) or cultured macrophages ($1x10^6$ cells/mL) was mixed with gelatin-Sepharose beads to purify secreted proMMP-9. Original CM, unbound fractions, and eluted proteins were analyzed in silver stained gels (top panels) or zymography gels (bottom panels). Triangles indicate the position of 92-kDa proMMP-9. Position of mol wt. markers in kDa is indicated on the left.

(C) Western blot analysis of proMMP-9 purified from different types of human myeloid cells. Neutrophil proMMP-9 (Neu) and proMMP-9 from monocytes (Mono) and macrophages of M0-, M1-, and M2-phenotypes was analyzed by SDS-PAGE under reducing conditions. Human recombinant proMMP-9 and TIMP-1 at amounts indicated above corresponding lanes (ng/lane) were run in the same gel to provide the means for quantification of molar ratios between proMMP-9 and proMMP-9-boind TIMP-1 in the fractions eluted from gelatin-Sepharose beads. Quantitative analysis of this particular blot indicates the ratios of 3.0, 1.0, 1.5, and 18.2 for monocytes, M0-, M1-, and M2-macrophages, respectively. Note that neutrophil proMMP-9 is completely devoid of TIMP-1.



<u>Supplemental Figure S2</u>. Analysis of CM from M0-macrophages for putative inhibitors of angiogenesis

CM from M0- and M2-macrophages was analyzed in CAM angiogenesis assay independently or in combination. Bar graph presents means ± SEM of fold changes in the levels of angiogenesis compared to SF control (1.0). ***, $P<0.0001$, in comparison with angiogenesis levels induced by M0-CM. The similar levels of angiogenesis induced by M2-CM and the M0+M2 mixture indicate that low levels of angiogenesis induced by M0-CM are not caused by putative angiogenesis inhibitors.



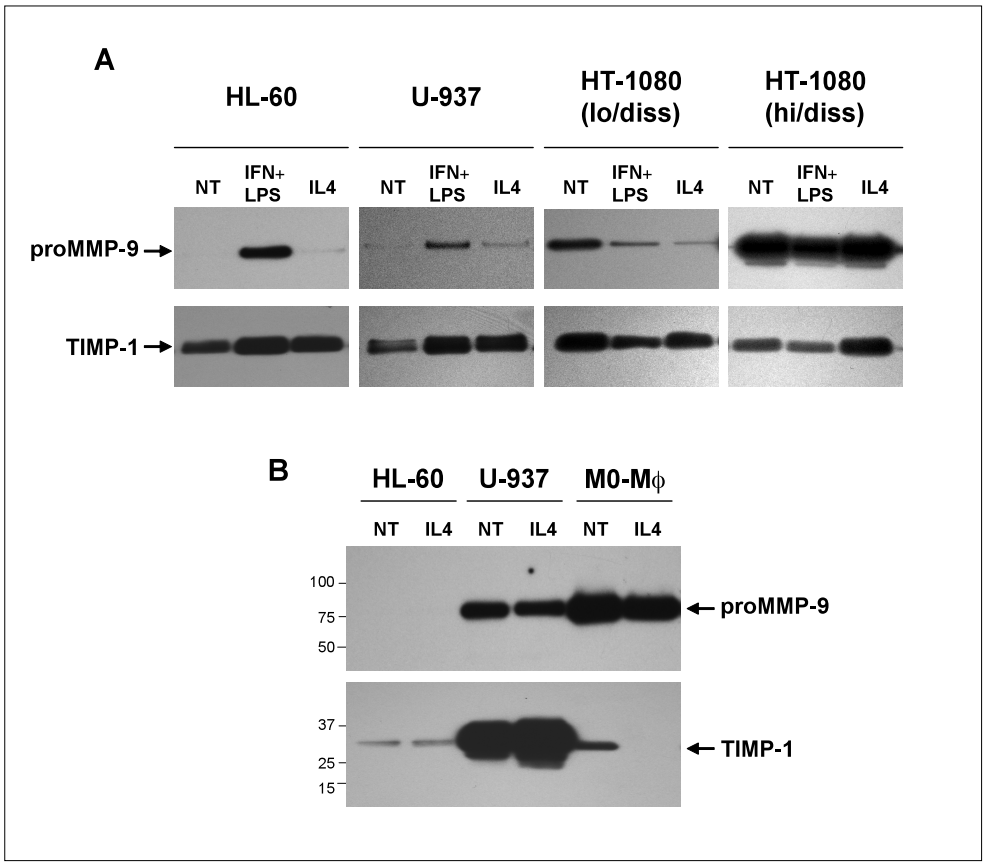
Supplemental Figure S3. Interleukin-mediated induction of M2-phenotype in human macrophages

Mature human macrophages (M0-phenotype) were incubated with 20 ng/mL of human recombinant IL-4, IL-10 or IL-13. Alternatively, M0-macrophages were treated with IFNγ/LPS to induce M1-polarization. Non-treated cells (NT) served as negative control. Following a 24-hr incubation, the cells were lysed and mRNA was extracted. Expression analysis was conducted for genes encoding α-arginase-1 (*ARG1*), iNOS (*NOS2*), and macrophage mannose receptor (*MRC1*) Presented are gene expression levels that were normalized to the levels of the housekeeping gene, *GUSB*, according to the formula $2^{-\Delta \text{ Ct target gene} - \text{Ct reference gene}}$.

(A) Gene expression analysis for α-arginase-1 demonstrates that *ARG1* is downregulated in M1-macrophages, but sustained in non-treated macrophages (M0-phenotype) and macrophages treated with IL-4, IL-10, and IL-13 (M2-phenotype). *, $P<0.05$ in Mann-Whitney test.

(B) Gene expression analysis for iNOs demonstrates that *NOS2* is specifically induced in macrophages polarized towards M1-phenotype, whereas remaining negligibly low in non-treated and all interleukin-treated macrophages. *, $P<0.05$ in Mann-Whitney test.

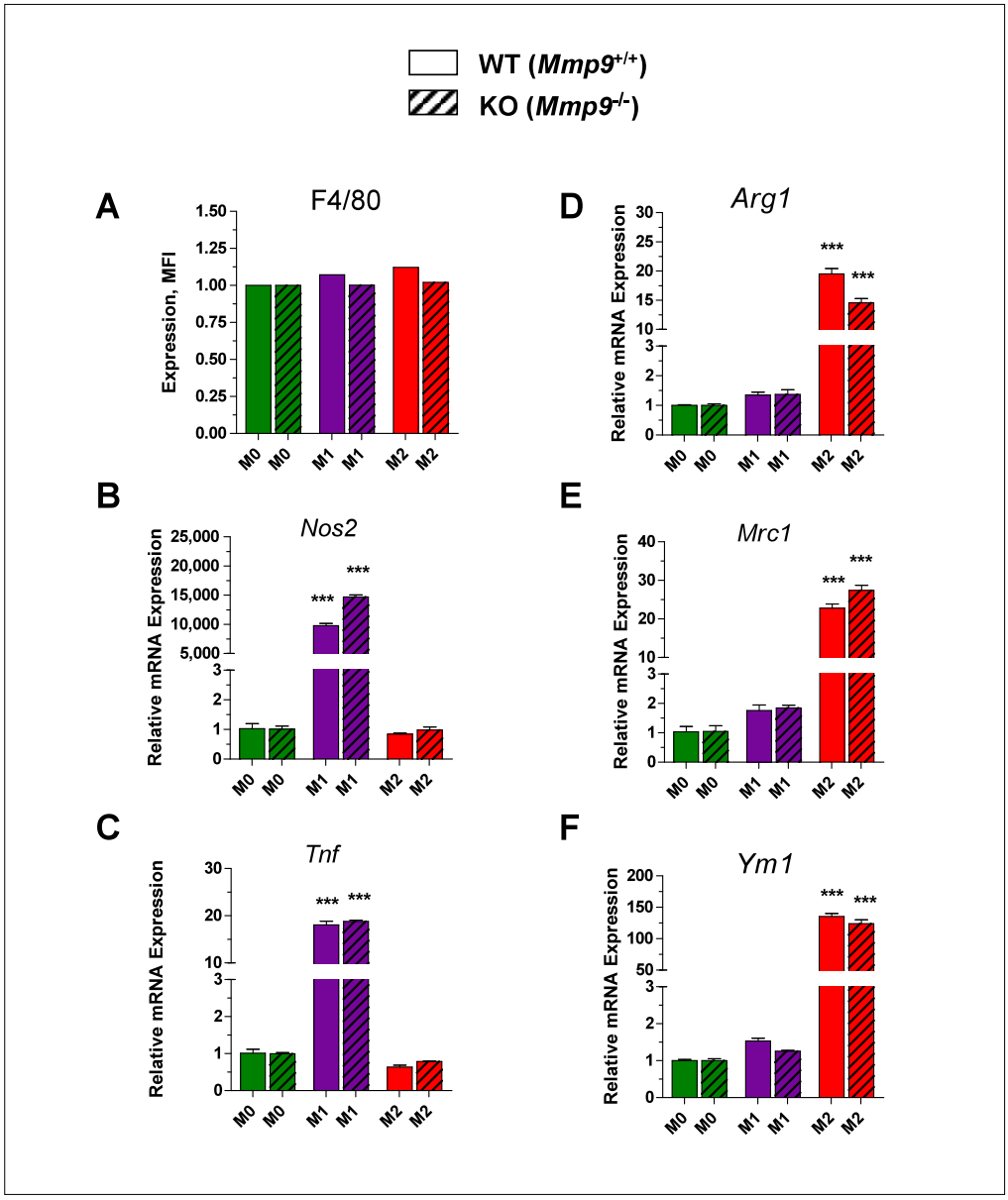
(C) Gene expression analysis for macrophage mannose receptor demonstrates that *MRC1* is downregulated in M1-macrophages, but sustained in non-treated macrophages (M0-phenotype) and macrophages treated with IL-4, IL-10, and IL-13 (M2-phenotype). ***, $P<0.0001$ in Student's *t*-test.



Supplemental Figure S4. Analysis of proMMP-9 and TIMP-1 expression in human leukemia and fibrosarcoma cells treated with IL-4

(A) Leukemia HL-60 promyeloblastic and U-937 histiocytic cells, and HT-1080 fibrosarcoma cells with high and low disseminating capacity (lo/diss and hi/diss), were treated with a mixture of IFNγ and LPS or IL-4 for 24 hr, and then transferred into SF-medium for 48 hr. Non-treated cells (NT) served as a negative control. CM was analyzed by western blotting for MMP-9 and TIMP-1. Volume of CM samples from different cell types and treatments was normalized to represent equal number of treated and non-treated cells ($2x10^4$). Although IFNγ/LPS and IL-4 have varying effects on proMMP-9 production depending on cell type, the levels of TIMP-1 were either slightly increased or remain the same in the treated compared to non-treated cells.

(B) IL-4-treated and non-treated (NT) HL-60 and U-937 cells were compared with IL-4-treated and non-treated M0-macrophages for production of proMMP-9 and TIMP-1. Volume of CM samples from different cell types and treatments was normalized to represent equal number of IL-4-treated and non-treated cells ($2x10^4$). Position of mol. wt. markers in kDa is indicated on the left. Importantly, IL-4 did not cause a decrease in TIMP-1 in HL-60 or U-937 cells, but completely shutdown TIMP-1 production in treated macrophages.

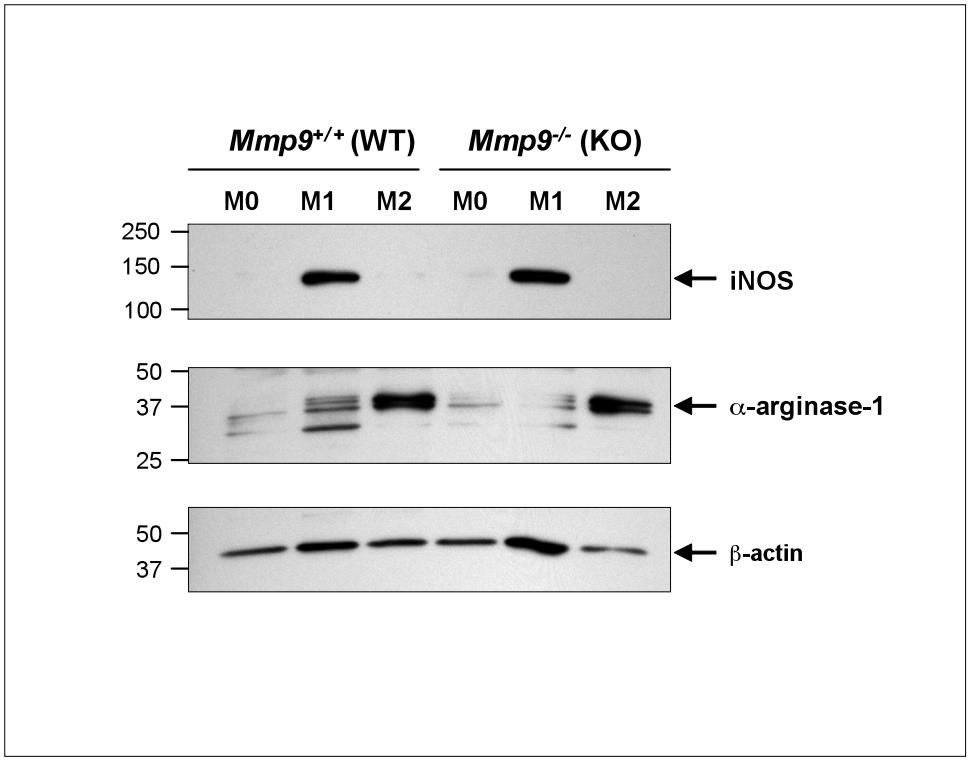


Supplemental Figure S5. FACS and gene expression analysis of murine bone marrow-derived macrophages

M0-macrophages were generated from WT (*Mmp9*$^{+/+}$) and *Mmp9* KO (*Mmp9*$^{-/-}$) C57BL/6 mice by incubating bone marrow cells in the presence of murine M-CSF for 7 days. BM-derived M0-macrophages of each genotype were polarized into M1- or M2-phenotypes in the presence LPS/IFNγ or IL-4, respectively. Following 48 hr, the cell layers were washed twice in PBS and processed for FACS analysis (**A**) or gene expression analysis (**B-F**).

(A) FACS analysis was performed on cells detached with enzyme-free buffer and stained with AlexaFluor488-conjugated rat mAb F4/80. The MFI was determined for each cell phenotype and then normalized against MFI of M0-cells of the corresponding genotype (expressed as 1.0).

(B-F) Gene expression analysis was performed on cDNA generated from mRNA extracted from the indicated cell phenotypes/genotypes, according to the manufacturer's instructions (Clontech). For each cell genotype/phenotype, the expression levels of indicated genes were normalized to the levels of β-actin, generating individual ΔCt values (Ct target gene – Ct β-actin). For different cell phenotypes, expression levels of individual genes were determined relative to M0 cells of the corresponding genotype (1.0), according to the formula $2^{-\Delta\Delta Ct}$ (ΔCt macrophage phenotype - ΔCt M0-phenotype). The genes that are associated with the M1-phenotype are *Nos2* (encoding iNOS) and *Tnf* (encoding TNFα) The genes associated with the M2-phenotype include *Arg1* (encoding α-agrinase-1), *Mcr1* (encoding macrophage mannose receptor, MMR), and *Ym1* (encoding ECF-L). ***, $P<0.0001$, in comparison with M0-phenotype; two-tailed unpaired Student's *t*-test.



Supplemental Figure S6. Western blot analysis of iNOS and α-arginase-1 in polarized murine BM-derived macrophages

M0-macrophages were generated from *Mmp9*$^{+/+}$ (WT) and *Mmp9*$^{-/-}$ (KO) C57BL/6 mice by incubating bone marrow cells in the presence of murine M-CSF for 7 days. BM-derived M0-macrophages of each genotype were polarized towards M1- or M2-phenotypes in the presence of IFNγ/LPS or IL-4, respectively. Following 48 hr, the cell layers were washed and lysed in mRIPA buffer. The proteins (20 μg per lane) were separated by SDS-PAGE in 4-20% gels and transferred onto a membrane support. Membranes were probed for iNOS (expected band of 130 kDa) and α-arginase-1 (expected band of 37 kDa). Re-probing for β-actin (expected band of 42 kDa) served as loading control.

References

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